QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION

Antibody Production in Rabbits Injected with an Azo Protein*

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The antibody titer of a serum has usually been given in terms of the highest dilution at which it will agglutinate, hemolyze, or precipitate the antigen, in terms of the volume of toxin it will neutralize, in terms of the optimal proportion in which flocculation with antigen takes place, in terms of mouse protection—all relative and often inaccurate measures giving no idea whatsoever of the actual mass of antibody involved. The dilution methods, particularly, are subject to an error of 50 to 100 per cent in the decision as to which tube is the last positive one.

For the quantitative measurement of precipitins one need no longer be dependent upon such methods. With the recognition of antibodies as modified serum globulins (1) and with the establishment by the writers of the conditions for their maximum precipitation (2) an absolute method for the estimation of precipitating antibodies has been worked out (3), based on the earlier use, by Wu and his coworkers (4), of the micro Kjeldahl method for the analysis of antigen-antibody precipitates. The first application of this method was in the standardization of Type I antipneumococcus horse sera (3), in which the writers, with Sia, showed a parallel between mouse protection and the maximum amount of specifically precipitable protein, a relation which was confirmed by Felton (5). A preliminary report was then made on the amounts of antibody produced following injection of a red azo

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dye, R-salt-azo-benzidine-azo-crystalline egg albumen (6). Culbertson has also used the method in a study of the crystalline egg albumen-antibody system and devised a modification suitable for that system (7). The method may also be reversed, and used for the micro determination of precipitating haptens such as specific polysaccharides (8), or for antigens. A similar method for the analysis of antigen-antibody precipitates has been employed by Marrack and Smith (9), while the optimal proportions method has been proposed both for the determination of antigen (10) and of antibody (11).

In the quantitative determination of antibody in an antigen-antibody system it is necessary to distinguish between two kinds of protein, since both antigen and antibody are, as far as is known, proteins. This problem was first solved in two ways by Wu and his collaborators (4) by using hemoglobin and iodoalbumen as antigens and determining their amount in the specific precipitate by appropriate methods. Unfortunately, Wu's studies extended over only a portion of the reaction range, and at the time the conditions for the maximum precipitation of antibody were not understood.

To the writers it seemed that the simplest way of distinguishing between antigen and antibody nitrogen (or protein) would be to employ colored antigens such as the azo proteins which had proved so valuable in the study of the chemical basis of specificity by Landsteiner (12) and by Avery and Goebel (13). For the purpose of a quantitative study of the relations between antigen and antibody in the precipitin reaction it was felt that a crystalline protein, such as egg albumen, should be used, and that it should be coupled with a more intensely colored dye than had been found necessary by these workers, in order that colorimetric determination of the antigen in precipitates or supernatants might be as accurate as possible. It was also felt that the azo antigen should be freed from components showing the original egg albumen specificity in order to eliminate the necessity of using a second azo antigen in the quantitative precipitin tests. A purplish red disazo dye, R-salt-azo-benzidine-azo-egg albumen, was finally isolated in a form which satisfied these requirements and was used in the quantitative studies on antibody production referred to above, and in a study of the mechanism of the precipitin reaction (6). Since this preliminary report azo proteins have been found useful in quanti-

tative work by Marrack and Smith (14) and by Breinl and Haurowitz (15).

The quantitative studies on the mechanism of the precipitin reaction between R-salt-azo-benzidine-azo-egg albumen and its homologous antibody will be reported in detail in a separate communication. The present paper deals with quantitative observations on precipitin production in rabbits following multiple injections of known amounts of the azo antigen.

EXPERIMENTAL

1. Preparation of the Azo Protein.—0.46 gm. of benzidine was dissolved in 100 cc. of water containing 3 cc. of 1:1 hydrochloric acid and tetrazotized at 7–8°C. with an aqueous solution of 0.35 gm. of sodium nitrite. The solution was poured into a chilled solution of 3 gm. of sodium acetate in 500 cc. of water, and to this was added a solution of 0.87 gm. of R-salt in 100 cc. of water. The R-salt was an especially pure preparation supplied through the kindness of Dr. M. L. Crossley of The Calco Chemical Company. The mixture slowly reddened, but the coupling was not completed until 20 cc. of normal potassium carbonate solution had been added. The intensely colored solution now contained tetrazotized benzidine coupled on one side with R-salt, but with the other diazo group free.

Three times recrystallized egg albumen (16) was dialyzed free from ammonium salts and 6 gm. of the protein in 1 liter of water at room temperature were made alkaline with 40 cc. of 2 N potassium carbonate solution. 100 cc. of the above diazo solution were then run in every 10 to 20 minutes as tests made by adding a few drops of the solution to carbonate-containing R-salt solution showed coupling to be complete. After a total of 600 cc. of diazo solution had been run in 20 cc. more of 2 N potassium carbonate solution were added. While egg albumen is capable of combining with somewhat more diazo solution than the amount actually used, it was considered advisable not to continue the coupling process to the limit, as in other cases this had resulted in insoluble complexes. Potassium carbonate was used since potassium salts of the diazo component and the coupled protein dye appeared to be less easily salted out at the above concentrations than did the sodium salts.

The dye protein solution was next chilled and acidified with acetic acid until flocculation first occurred, the optimum pH range varying from 4.6 to 4.2 in different preparations. The bicolor standard method was used (17), adding blank tubes of the pink supernatant. In this way good readings could be obtained with bromocresol green. The main supernatant, on acidification with more acetic acid, yielded less highly colored material, and this was either discarded or added to a subsequent preparation.

The crude R-salt-azo-benzidine-azo-egg albumen was collected by centrifugation, dissolved in about 750 cc. of water with the minimum amount of N sodium

carbonate solution, centrifuged to remove a small amount of violet-colored insoluble material, and again acidified with the minimum amount of acetic acid required for flocculation.[1] In this way small amounts of less highly colored protein, reactive with anti-egg albumen serum, generally remained in solution, so that under optimal conditions 20 to 25 repetitions of the process resulted in recovery of the main portion of the azo protein as a clear, purplish red solution which no longer precipitated anti-egg albumen serum.[2]

In order to remove non-protein dye impurities the mixture was centrifuged as sharply as possible after the fifth or sixth precipitation and stirred in a freezing mixture with chilled acetone for ½ hour. After centrifugation (always in the cold) the dark red supernatant was discarded and the precipitate taken up in cold water and redissolved and reprecipitated as before. The acetone treatment usually resulted in denaturation of a small portion of the dye.

In one preparation 17 reprecipitations with acid sufficed to reduce the content of substances reacting with anti-egg albumen serum so that solutions as strong as 0.2 per cent failed to precipitate the serum. However, sera of rabbits immunized with the dye are precipitated by suitable concentrations of egg albumen. Quantitative experiments have indicated that it is not anti-egg albumen, but antibody to the dye which is precipitated by egg albumen from these sera. The details will be presented separately.

In another preparation 25 reprecipitations failed to remove impurities reactive with anti-egg albumen serum. The solution was then adjusted to 0.5 per cent phenol concentration and allowed to stand over the summer in the ice box, freezing solid during the period. After the ice had been melted it was found that much of the azo protein had been denatured. The entire mixture was diluted to 1.5 liters, stirred for 2 hours at room temperature after addition of 75 cc. of N sodium carbonate solution, stirred 2 hours longer in the cold, and was finally centrifuged. The precipitate was again extracted with very dilute sodium carbonate solution and the insoluble residue was discarded. The extracts were precipitated with acetic acid and solution and reprecipitation repeated five times, after which the dye, when redissolved, no longer reacted with anti-egg albumen serum. It was also possible in this case to sterilize the solution by filtration through a Berkefeld V candle, a process which had been unsuccessfully attempted with the preceding batch.

As a final step, the dye solution was ultrafiltered in the ice box through a collodion membrane and repeatedly washed with sterile 0.9 per cent saline. The washings were colorless at first, owing to adsorption of the dye by the porous earthenware support of the membrane, but eventually much colored material passed through and this was not precipitable by acetic acid or by antiserum to the dye. Washing was discontinued when the color of the filtered solution became

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[1] All operations were conducted in the cold, and a refrigerating centrifuge, manufactured by the International Equipment Company, Boston, was used.

[2] Four sera of high antibody content, kindly supplied by Dr. J. T. Culbertson, yielded traces of precipitate after centrifugation in the cold.

less intense than that of a 1:100,000 solution of the dye. The filter contents were then centrifuged to remove traces of violet precipitate and the dye solution was standardized by analysis for nitrogen by the micro Kjeldahl method. The azo protein was found to contain 14.6 per cent of nitrogen on the ash-free basis. The solution was kept sterile in the ice box by the occasional addition of a drop of chloroform.

2. Immunization of Rabbits with the Azo Protein.—In all but one series the injections were given intravenously. Four daily injections were given each week for 4 weeks, after which bleedings were made on the day indicated. Many animals were given additional courses of 2, 3, or 4 weeks. In only a few instances were more than 2 or 3 mg. injected at one time. The procedure used for each group of animals is summarized in the tables.

Material for the experiments summarized in Table II was obtained as follows: 1.5 cc. of a solution of the ultrafiltered azo protein containing 13.8 mg. were diluted to 10 cc. and treated with 0.08 cc. of 0.1 N acetic acid, an amount insufficient to precipitate the dye but adequate to increase the amount adsorbed by collodion particles over the amount taken out in neutral solution. The dye solution was rinsed into a suspension of collodion particles which had been prepared in the usual way by dilution of an acetone solution of collodion with water and rejection of the coarse particles. The mixture was stirred occasionally during several hours, allowed to stand overnight in the ice box, and was then centrifuged. The supernatant was poured into a volumetric flask and to it were added the next two washings with water. A third washing with saline extracted no more dye. The amount of dye left in the supernatant was determined colorimetrically by comparison with a solution of known concentration, giving, by difference, the amount adsorbed on collodion. The suspension was then diluted with saline (plus 0.01 per cent merthiolate) so that 50 cc. of suspension, the amount used for 16 injections, contained 0.55 mg. of dye. The initial dose was 1.0 cc. and this was gradually increased, so that at the end the rabbits each received 5.0 cc. Eight animals were injected in this way. The 50 cc. of suspension contained 140 mg. of collodion.

The remaining animals which received smaller amounts of collodion-adsorbed dye were in earlier series.

For comparison with the eight animals receiving 50 cc. of the collodion suspension, five rabbits were treated with identical doses of a *solution* of the dye at the same dilution, and five more with a *suspension* of the same concentration containing 14 per cent by volume of 1 per cent alum solution (18).

In the last three lines of Table II are summarized the data on a number of rabbits injected with stronger solutions and more concentrated alum suspensions of the azo protein.

3. Determination of the Maximum Amount of Precipitable Antibody in the Sera.—In the original method (3) the precipitate formed by the specific polysaccharide of Type I pneumococcus and its homologous antibody was washed only once with a 1:20,000 solution of specific polysaccharide in saline. It has since been found (8) unnecessary to add the specific carbohydrate to the washing fluid, nor does disso-

ciation occur in the azo protein-antibody system when the precipitate is washed in the cold with 0.9 per cent saline alone. Two washings are also necessary (8) if complete removal of non-specific serum proteins is desired. The method follows as applied in the present instance:

Depending on the intensity of a preliminary rapid qualitative test with 1:10,000 dye, amounts of serum ranging from 0.5 to 4.0 cc. are used. A number of sera may be analyzed at one time. The sera should be measured in duplicate with accurately calibrated pipettes into wide agglutination tubes (10 mm. inside diameter x 75 mm.) or Wassermann tubes, depending on the amount of serum used. Blanks should also be run in duplicate and saline added to these instead of dye solution. If less than 2 cc. of serum have been used the volume should be made up to 2 cc. with saline. An amount of a 1:1000 solution of the dye in saline is then added sufficient to provide a *slight* excess of the dye. Not more than 0.10 cc. should be added in the case of sera containing less than 0.1 mg. of precipitable antibody per cc. (3 to 4 cc. samples), and the volume of stronger sera should be chosen so that not more than 0.2 or 0.3 cc. of the 1:1000 dye solution need be used. Calibrated pipettes are not necessary for the dye. The tubes are plugged and the contents carefully and thoroughly mixed by a rotary motion imparted by drawing the fingertips rapidly and repeatedly diagonally down the side of the tube. The tubes are set in the water bath and may be centrifuged, if desired, as soon as the precipitate begins to settle, in order to make sure that an excess of dye has been added. If the supernatant is not definitely pink as compared with a blank on the same serum, 0.05 to 0.1 cc. more of the dye dilution should be added at a time until a definite excess is present. Supernatants should not contain so large an excess as to be definitely red, as many sera show a marked inhibition zone beginning with surprisingly low concentrations of antigen in excess. If the supernatants are red, more serum should be added to the determinations and blanks, or a new analysis started. In the case of weak sera the precipitates are often very slow in forming. The tubes are allowed to stand 2 hours in the water bath at 37° and overnight in the ice box, or else may be left at room temperature for a period and then overnight in the ice box, or may be immediately put into the ice box for 15 to 18 hours. The rabbit antisera tested in this laboratory have given identical results under these conditions, except that the precipitate forms at a slower rate in the cold. The systems tested have been dye-antidye, egg albumen and its homologous antibody, and Type III pneumococcus specific polysaccharide and rabbit anti-Type III pneumococcus serum, so that in these cases there would seem to be no basis, except on the ground of increased speed of reaction, for the current immunological practice of allowing precipitin tests to stand 2 hours at 37° before placing in the ice box overnight. This observation does not apply to immune horse sera, in which the differences found are being subjected to closer study.

After the tubes have stood overnight they are centrifuged in the refrigerating centrifuge or in carriers containing ice water for 15 to 20 minutes at about 1500 revolutions per minute. The supernatants are then carefully decanted and the

tubes are inverted, allowed to drain 5 minutes, and the mouths wiped with filter paper. The tubes are placed in ice water and 0.5 cc. of ice-cold saline is added to each. The contents are mixed as before and the red precipitates should be thoroughly disintegrated in order to insure as complete removal of non-specific protein as possible. The tubes are then rinsed down with 1.5 cc. of ice-cold saline in the case of the small tubes and 2.5 cc. for the Wassermann tubes and again mixed.

Blank tubes which show no whirl when the contents are mixed at the first or second washing may be discarded, as under these conditions the blank to be deducted in the nitrogen determination is no larger than that on the reagents alone.

While the tubes are standing in ice water for ½ hour the original supernatants should be tested as a control for the presence of a slight excess of dye. To one 0.5 cc. portion of the mixed duplicate supernatants from each serum is added 0.1 cc. of a 1:10,000 dye solution, to another, 0.2 to 0.3 cc. of the blank supernatant from a serum which has given a heavy precipitate. There should, of course, be no precipitate in the tube to which additional dye was added, while the tube to which antiserum was added should show a slight turbidity within 2 hours or at least a slight precipitate on standing overnight in the ice box.

After ½ hour in the cold the washed precipitates are centrifuged, decanted, and drained as before, and again washed as above with 1.5 or 2 cc. of chilled saline, depending on the size of tube. After standing for ½ hour in ice water, the tubes are finally centrifuged, decanted, and drained. The precipitates are covered with 1.5 to 2 cc. of water, loosened from the bottom of the tube by rotating as before, and dissolved by the addition of 2 to 3 drops of N sodium hydroxide solution.

The amount of azo protein in the precipitate may be determined by making up the volume of the solution to 5.0 or 10.0 cc. and comparing the color with that of a known solution of the dye containing the same amount of alkali and a few milligrams of added colorless protein. Since the ratio of dye to antibody in the precipitate has been found in this laboratory to average 1:7 at the equivalence point (for definition see (2, 6, 1 *b*)), the determination of antigen in the precipitate may be omitted if it is desired to accept this figure. In the application of the method to any other antigen it would be necessary to determine this ratio for the system used (*cf.*, for example, (7)).

The solution of the precipitate is rinsed quantitatively into a micro Kjeldahl flask and the nitrogen determined by any standard procedure. The Pregl method, slightly modified, was used in the present work. Nitrogen found $\times$ 6.25 = specifically precipitated protein in the sample. Total protein minus antigen protein $\left(\frac{\text{total}}{\cdot 8}\right)$ = antibody. For the determination of the total amount of circulating antibody in the animal the blood volume was taken as 5.5 per cent of the weight at the time of bleeding, according to Meek and Gasser (19), and the serum volume as one-half of the blood volume.

DISCUSSION

1. Precipitin Content of Rabbit Sera as Influenced by the Period between Final Injection and Bleeding.—In Table I is given a summary of antibody determinations on sera taken at various periods after the

TABLE I

Influence of Days between Last Injection and Bleeding, also of Repeated Bleedings, on Precipitin Content of Rabbit Sera

Rabbit No.	Total antigen injected	Total No. of injections	Weight precipitable antibody per cc. on day indicated after last injection						
			3rd	4th	5th	6th	7th	8th	10th
	mg.		mg.	mg.	mg.	mg.	mg.	mg.	mg.
	In solution								
5-5	18.7	18*				0.97		0.75	
6-8	13.2	16				1.22	1.04		
2nd course									
5-5	26.4	25*	0.79	0.61	0.39				
5-6	26.4	25*	0.36	±†	±†				
6-2	26.4	25*	0.83	0.85	0.47				
6-4	20.9	23	0.66	0.62					
6-8	20.9	23	2.06	1.88	2.04				
6-9	20.9	23	1.09	0.88	1.08				
3rd course									
5-5	53.9	32	±		0.79		0.81		0.89
6-1	53.9	32	±		0.94		0.86		0.80
6-2	53.9	32	±		0.42		0.36		
	As alum precipitate								
8-9	14.5	18	2.03	1.91					
1-03	36.1	19			1.43	1.48			
1-12	28.4	32			0.95		0.75		
1-15	19.1	30			1.87 (test bleeding)				
					1.58 (main bleeding, same day)				
1-20	28.1	16			0.46		0.43		
	On collodion particles								
1-31	0.55	16			0.24		0.24		
1-33	0.55	16			0.54		0.23		

* All but two injections subcutaneous.

† Traces of precipitate in the 0.5 cc. samples used.

final injection of azo protein. 3 days was the shortest interval and 10 days the longest. It is apparent that the maximum antibody content

was usually reached by the 3rd day after the last injection. In one series, in which unusually large doses of 6.7 mg. had been given at the end, the sera yielded only traces of precipitate on the 3rd day.

It is also clear that with these exceptions and in the case of one 10th day bleeding in the same series[3] of animals, the maximum titer obtainable was contained in the first bleeding. In subsequent bleedings within 1 or 2 days different animals varied greatly in the antibody content of the serum yielded, some showing great constancy, and others a gradual or irregular diminution. In one case (No. 1-15) the serum from a small initial bleeding of not more than 5 cc. contained 1.89 mg. of precipitable antibody per cc., while the serum from the main bleeding, only several hours later, contained but 1.58 mg. per cc.

As a result of these tests it is the custom in this laboratory to bleed animals 5 or 6 days after the last injection, although any day from the 4th to the 10th would probably serve as well.

2. Experiments Summarized in Table II.—All of the 18 rabbits injected intravenously with 0.55 mg. of the azo antigen in multiple doses either in solution, as an alum precipitate, or adsorbed on collodion particles, responded with measurable amounts of precipitin. Four rabbits which received as little as 0.35 mg. on collodion particles also responded well. The lower limit for the amount of azo protein capable of stimulating precipitin formation appears to be about 0.2 mg. in multiple doses, for of 10 rabbits receiving 0.15 to 0.23 mg. on collodion particles, only two showed precipitins. This is in accord with the findings of Hektoen and Cole (20), who placed the lower limit for egg albumen at about 0.3 mg.

The magnitude of the antibody response to multiple minimal doses of the antigen is indeed surprising. It has, of course, long been known in a qualitative way that antibody production is in excess of the amount required to combine with the antigen, and this fact provided Ehrlich with a strong argument against the Buchner hypothesis of antibody formation. Quantitative data on this point are now made available in Table II, from which it is seen that as much as 0.73 to

[3] Most of the analyses in this series and the one preceding it were carried out on 0.5 cc. samples in order to keep the individual bleedings as small as possible. The difference between the 5th and 10th day bleedings is therefore within the limit of error on so small a sample of a serum of comparatively low titer.

0.94 mg. of circulating antibody per cc. of serum may be formed in response to injections of antigen totaling 0.35 to 0.55 mg., or a total response for the rabbit of over 100 mg. of circulating antibody for every milligram of antigen injected. This is at least 12 times as much as is necessary to combine with the amount of antigen used. There is also an appreciable amount of antibody in the tissues as

TABLE II

Precipitin Formation in Rabbits Following Multiple Injections of R-Salt-Azo-Benzidine-Azo-Crystalline Egg Albumen

No. of rabbits	Total dye injected into each	Total No. of injections	Precipitable antibody per cc.	Total circulating precipitin	Maximum amount circulating precipitin formed per mg. injected
	mg.		*mg.*	*mg.*	*mg.*
5	0.55 (solution)	16	0.06–0.53	4–44	80
5	0.55 (alum)	16	0.28–0.73	25–51	93
8	0.55 (collodion particles)	16	0.07–0.86	5–60	109
4	0.35 (collodion particles)*	16	0.16–0.73	10–38	109
4	0.23 (collodion particles)	11	0, 0, 0, 0.04	0–3	13
4	0.15 (collodion particles)	14	0, 0, 0, 0.10	0–8.5	57
	Experiments with Larger Doses				
5	0.55† + 3.1 (alum)	22	0.51–1.53	29–109	30
23	9.2 − 36.1 (alum)	16–33	0, 0, 0, 0.17–3.39	0–203	21
10	12.9 − 26.4 (solution)	13–25	0, 0, 0.50–2.08	0–129	6

* After three more injections with an additional 0.1 mg. of dye on collodion particles the serum of the best animal contained 0.94 mg. of precipitable antibody per cc., or a total of 49 mg. for the entire circulating precipitin (No. 1-14, Table IV).

† This group consisted of the five best antibody producers among 15 rabbits in the first three groups.

well, and the presence of antibodies other than precipitins is not excluded. Thus the relative mass of antibody formed is so large as to be taken as supplementing other recent evidence (1 *b*) against Buchner's hypothesis of the actual entrance of specific antigen fragments into the antibody molecule.

A summary is also given in Table II of precipitin formation in 10

rabbits which received larger doses of the azo protein in solution, and in two series totalling 28 rabbits which received the dye as the alum precipitate. The total amounts of azo protein injected ranged from 3.7 to 35 mg., and it is seen that while the maximum relative response is lower with the larger doses, the total amount of precipitin produced is often higher. As stated by Hektoen (21) there is no relation between the dosage and the amount of precipitin produced.

Sixteen sera contained more than 1 mg. of precipitin per cc. Thirteen of these were in the alum group, as was the serum of highest titer; namely, 3.39 mg. per cc. This serum contained 75 mg. of protein per cc. (calculated from total nitrogen), so that in this rabbit 4.5 per cent of the serum protein consisted of antibody.[4]

Five of the rabbits, of which three were in the alum group, failed to show qualitative or quantitative evidence of circulating precipitins, further emphasizing the enormous individual variations in the immunological response of rabbits. In spite of these individual differences it would seem fair to give the preference to multiple intravenous injections of the dye as the alum precipitate, since sera of higher antibody content were obtained more regularly when the dye was used in particulate form. The use of the dye adsorbed on collodion particles is naturally limited to very small doses.

After the final bleeding one rabbit (No. 1-57) which had received 0.55 mg. of the dye adsorbed on collodion particles and one which had received 35.1 mg. of the alum precipitate (No. 1-32) were autopsied. The spleens and portions of the livers were fixed in bichloride-acetic acid solution and unstained sections were made. These were kindly examined by Dr. Franklin M. Hanger, Jr., of this Department, who found, in the first animal, collodion particles in the Kupffer cells and in the phagocytic cells of the reticulum of the spleen. In the second animal azo protein particles could be seen in a few of the same groups of cells (*cf.* also (22)).

[4] The highest antibody titer observed in this laboratory occurred in the serum of a rabbit given repeated small intravenous injections of formalinized Type III pneumococcus vaccine. The amount of protein specifically precipitable from this serum by the Type III pneumococcus specific polysaccharide was 17.0 mg. per cc. Calculated from the total nitrogen, the serum contained 91 mg. of protein per cc. Thus more than one-sixth of the serum protein was anticarbohydrate. Antipneumococcus sera titering 7 to 9 mg. per cc. of anticarbohydrate were frequently encountered (see also (3)).

The ability of a rabbit to build up antibodies to the azo protein failed to serve as an index of the animal's response to stimulation with another antigen such as Pneumococcus or Streptococcus. A number of excellent antibacterial sera were obtained from rabbits which had given antidye sera of low precipitin content. Conversely, Rabbits

TABLE III

Precipitin Content of Rabbit Sera as Influenced by Repeated Courses of Injections of Antigen Solution

Rabbit No.	Course of injections	Total antigen injected	Total No. of injections	Precipitable antibody per cc.	Day of bleeding
		mg.		*mg.*	
5-5	1	18.7	18	0.97	6
	2	26.4	25	0.79	3
	3	53.9	32	0.89	10
5-6	1	18.7	18	0.55	6
	2	26.4	25	0.36	3
6-4	1	13.2	16	0.62	6
	2	20.9	23	0.66	3
6-8	1	13.2	16	1.22	6
	2	20.9	23	2.06	3
	3	53.9	32	0.52	5
	4	88.0	48	2.30	4
6-9	1	13.2	16	0.77	6
	2	20.9	23	1.09	3
	3	53.9	32	0.39	5
5-2	1	18.7	18	0.63	6
	2	26.4	25	±*	5
6-2	1	18.7	18	±*	6
	2	26.4	25	0.85	4

* Traces of precipitate.

6-8 and 6-9 yielded antidye sera of average precipitin content but gave very little anticarbohydrate when subsequently injected with Pneumococcus III.

3. Variations in Precipitin Content of Sera of Rabbits Given More than One Course of Injections.—The data are summarized in Tables III

and IV. For the sake of brevity only the principal types of behavior observed are recorded. Perhaps the most frequently encountered types of variation were (1) that in which the rabbit reached a relatively

TABLE IV

Precipitin Content of Rabbit Sera as Influenced by Repeated Courses of Injections of Azo Protein in Particulate Form

Rabbit No.	Course of injections	Total antigen injected	Total No. of injections	Precipitable antibody per cc.	Day of bleeding
		mg.		*mg.*	
1-03	1	0.23 (collodion particles)	11	0.04	6
	2	36.1 (alum)	19	1.48	6
	3	74.4 (alum)	32	1.43	5
1-04	1	0.23 (collodion particles)	11	0	6
	2	36.1 (alum)	19	±*	6
	3	76.5 (alum)	33	0.52	3
1-14	1	0.35 (collodion particles)	16	0.73	4
	2	0.45 (collodion particles)	19	0.94	4
	3	9.2 (alum)	25	3.39	5
	4	19.1 (alum)	33	3.33	5
1-33	1	0.55 (collodion particles)	16	0.54	5
	2	3.6 (alum)	22	0.51	6
	3	6.2 (alum)	29	0.51	6
	4	8.9 (alum)	37	0.33	6
1-40	1	0.55 (alum)	16	0.71	5
	2	3.6 (alum)	22	1.48	6
	3	6.2 (alum)	29	0.36	6
	4	8.9 (alum)	37	0.77	6
1-46	1	0.55 (solution)	16	0.53	5
	2	3.6 (alum)	22	1.53	6
	3	6.2 (alum)	29	1.53	6
	4	8.9 (alum)	37	1.42	6

* Traces of precipitate.

low but maximum antibody content with the first course, and (2) a precipitin content which increased with successive courses to a maximum. For most rabbits there appeared to be a maximum number of

milligrams of precipitin which the individual could produce as a result of stimulation with repeated doses of the azo protein, whether the amount used in a single dose were a small fraction of a milligram or as high as 8 mg.

4. Stability of Antisera.—In Table V are given the original precipitin content of a number of sera and the number of milligrams found per cubic centimeter after periods up to 9 months in the refrigerator. The tubes were covered with rubber caps. Sera 1-12 to 1-20 contained 0.01 per cent of merthiolate. Only in the case of three sera was the loss

TABLE V

Influence of Storage on the Precipitable Antibody of Rabbit Sera

Rabbit No.	Weight precipitable antibody per cc. at time of bleeding	Interval between determinations	Precipitable antibody per cc. after storage
	mg.	*mos.*	*mg.*
6-0	1.06	7	0.99
6-8	2.04	5.5	1.48
6-9	1.08	5.5	1.04
8-9	1.92	4.5	1.42
9-3	0.83*	9	0.83
1-12	0.75	6	0.67
1-14	3.33	6	3.06
1-15	1.59	6	1.53
1-16	1.02	6	0.99
1-17	0.65	6.5	0.59
1-18	0.70	6.5	0.76
1-19	1.29	6.5	1.21
1-20	0.43	6.5	0.40

* This analysis was made 3 months after the bleeding.

in precipitin outside the limits of error of the method. It appears to be a widespread belief that antiprotein sera rapidly lose their antibody content, but the antisera to the azo protein in question certainly afford no support for this belief. Several of the sera are being preserved in sealed tubes and it is hoped to analyze them after a longer period.

SUMMARY

1. The preparation is described of a deep red protein dye, R-salt-azo-benzidine-azo-crystalline egg albumen, which contains no more than traces of protein with the original egg albumen specificity.

2. Based on previous publications of the writers, a quantitative method is given for the micro estimation of precipitin in the antisera to the dye. The method gives the actual weight of precipitin and may be applied to the determination of the maximum amount of precipitable antibody in any antiserum.

3. Data are given (1) on the influence of the period between the final injection and the bleeding on the precipitin content of rabbit antisera to the azo protein; (2) on the magnitude of the antibody response following the injection of multiple doses of the antigen varying within wide limits; (3) on the variations in the precipitin content of the sera of rabbits given successive courses of antigen injections; and (4) on the stability of antisera stored in the cold.

4. Four antisera were obtained in which over 100 times as much precipitin was recovered as the amount of antigen injected. This supplements the growing mass of evidence against the theory that specific antigen fragments are actually incorporated into the antibody molecule.

BIBLIOGRAPHY

1. *Cf.* (*a*) Wells, H. G., The chemical aspects of immunity, New York, Chemical Catalog Co., 2nd edition, 1929; (*b*) Heidelberger, M., in Luck, J. M., Annual review of biochemistry, Stanford University, Stanford University Press, 1932, **1**, 655; 1933, **2**, 503.
2. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1929, **50**, 809.
3. Heidelberger, M., Sia, R. H. P., and Kendall, F. E., *J. Exp. Med.*, 1930, **52**, 477.
4. Wu, H., Cheng, L. H., and Li, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1927, **25**, 853. Wu, H., Sah, P. P. T., and Li, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1929, **26**, 737.
5. Felton, L. D., *J. Immunol.*, 1931, **21**, 341.
6. Heidelberger, M., and Kendall, F. E., *Science*, 1930, **72**, 252, 253.
7. Culbertson, J. T., and Seegal, B. C., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 909. Culbertson, J. T., *J. Immunol.*, 1932, **23**, 439.
8. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55**, 555.
9. Marrack, J., and Smith, F. C., *Proc. Roy. Soc. London, Series B*, 1930, **106**, 1; *Brit. J. Exp. Path.*, 1930, **11**, 494; 1931, **12**, 30.
10. (*a*) Dean, H. R., and Webb, R. A., *J. Path. and Bact.*, 1928, **31**, 89. (*b*) Taylor, G. L., Adair, G. S., and Adair, M. E., *J. Hyg.*, 1932, **32**, 340.
11. *Cf.* Reference 10 *a*, and Smith, W., *J. Path. and Bact.*, 1932, **35**, 509.
12. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1932, **56**, 399; and earlier papers.

13. Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1931, **54,** 431; and earlier papers.
14. Marrack, J., and Smith, F. C., *Brit. J. Exp. Path.*, 1931, **12,** 182.
15. Breinl, F., and Haurowitz, F., *Z. physiol. Chem.*, 1933, **214,** 111.
16. Prepared according to Heidelberger, M., Advanced laboratory manual of organic chemistry, New York, Chemical Catalog Co., 1923, 83.
17. Hastings, A. B., Sendroy, J., and Robson, W., *J. Biol. Chem.*, 1925, **65,** 381.
18. For this use of alum see Glenny, A. T., Pope, C. G., Waddington, H., and Wallace, U., *J. Path. and Bact.*, 1926, **29,** 38; and Glenny, A. T., and Waddington, H., *J. Path. and Bact.*, 1928, **31,** 413.
19. Meek, W. J., and Gasser, H. S., *Am. J. Physiol.*, 1918–19, **47,** 302.
20. Hektoen, L., and Cole, A. G., *J. Infect. Dis.*, 1932, **50,** 171.
21. Hektoen, L., *J. Infect. Dis.*, 1914, **14,** 404.
22. Smithburn, K. C., and Sabin, F. R., *J. Exp. Med.*, 1932, **56,** 867; especially page 886.